Anemia is probably one of the most well-known toxic effects of lead. Previously, lead-induced anemia was considered to be from the inhibition of δ-aminolevulinic acid dehydratase participating in the heme biosynthesis. However, little is known whether lead could affect the destruction of erythrocyte, another important factor for anemia. In the present study, we demonstrated that lead could accelerate the splenic sequestration of erythrocytes through phosphatidylserine (PS) exposure and subsequently increased erythropagocytosis. In freshly isolated human erythrocytes, Pb²⁺-induced PS exposure at relatively low concentrations (≤0.1μM) by inhibiting flippase, a key aminophospholipid translocase for the maintenance of PS asymmetry and adenosine triphosphate depletion appeared to underlie this phenomenon. Abnormal shape changes of erythrocytes and microvesicle generation and other triggers for the erythropagocytosis were also observed in the Pb²⁺-exposed erythrocytes. In vitro data showed that human macrophage indeed recognized and phagocytosed PS-exposed erythrocytes. In good accordance with these in vitro results, the oral administration of Pb²⁺ increased PS exposure on erythrocytes in rat in vivo. In addition, reduction of hematocrit and hemoglobin and increased spleen weight were observed along with enhanced splenic sequestration of erythrocytes in the rats exposed to Pb²⁺ subchronically for 4 weeks through drinking water. In conclusion, these results suggest that Pb²⁺-induced anemia may be explained at least in part by increased PS exposure on erythrocytes, erythropagocytosis, and splenic sequestration.

Key Words: erythrocyte; lead; anemia; phosphatidylserine exposure; phagocytosis.
underlying the lead-induced hemolytic anemia. However, there is no report on the effect of lead on erythropagocytosis, another important pathway for erythrocyte destruction to our best knowledge. Erythropagocytosis is the main route of normal and pathophysiological clearance of damaged erythrocytes (Otogawa et al., 2007; Waitumbi et al., 2000). Tissue macrophages in the reticuloendothelial system of spleen and liver recognize abnormal erythrocytes through various mechanisms. Erythrocytes with reduced deformability or abnormal shapes could be easily engulfed by macrophages (Fens et al., 2010). Recent interests are given to the role of phosphatidylserine (PS) exposure on erythrocytes in the erythropagocytosis (Kobayashi et al., 2007; Noh et al., 2010). PS, normally retained on the inner leaflet, becomes externalized to the outer membrane when exposed to various stimuli. PS on the outer membrane of erythrocytes is recognized by tissue macrophages as a signal for phagocytosis. In addition, PS-bearing microvesicles (MV) which are released from PS exposing erythrocytes can be engulfed by macrophages.

In the present study, we discovered that low concentrations of lead (Pb²⁺) induce PS exposure and MV generation in freshly isolated human erythrocytes. We elucidated the mechanism underlying and investigated if lead-induced PS exposure in erythrocytes can trigger phagocytosis and contribute to accelerated clearance by spleen and liver, employing in vitro and in vivo models in an effort to give a new insight into lead-induced anemia.

MATERIALS AND METHODS

Chemicals

Lead(II) acetate, calcium chloride, glutaraldehyde solution, osmium tetroxide, calcium ionophore A23187, bovine serum albumin (BSA), N-[hydroxyethyl]-piperazine-N‘-[2-ethanesulfonic acid] (HEPES), sodium citrate, dimethyl sulfoxide, trichloroacetic acid (TCA), Tris-base, Tris-acetate, poly-L-lysine, adenosine triphosphate (ATP) bioluminescent assay kit, phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Chemical Co. (St Louis, MO). 1-Palmitoyl-2-triphosphate (ATP) bioluminescent assay kit, phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Chemical Co. (St Louis, MO). 1-Palmitoyl-2-triphosphate (ATP) bioluminescent assay kit, phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Chemical Co. (St Louis, MO). 1-Palmitoyl-2-triphosphate (ATP) bioluminescent assay kit, phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Chemical Co. (St Louis, MO).

Flow Cytometric Analysis of PS Exposure and MV Generation

After erythrocytes were treated with deionized water (DW) (vehicle) or Pb²⁺ for 24 h at 37°C, aliquots of blood samples were diluted 10-fold with annexin-binding buffer (125mM NaCl, 10mM HEPES, pH 7.4) and further incubated with following substances for 30 min at room temperature in dark. Annexin V-FITC was used as a marker for PS detection, whereas anti-glycoprophorin A-RPE was used as an identifier of erythrocytes and erythrocyte-derived MVs. Negative controls for annexin V binding were stained with annexin V-FITC in the presence of EDTA instead of CaCl₂ adjusted to final 2.5mM. Samples were analyzed on a flow cytometer FACScalibur (Becton Dickinson, San Jose, CA) equipped with argon ion laser emitting at 488 nm. The light scatter and fluorescence channels were set on log scale. Data from 10,000 events were collected and analyzed using CellQuest Pro software.

Microscopic Observation Using Scanning Electron Microscopy and Confocal Microscopy

After fixation with 2% glutaraldehyde solution for 1 h at 4°C, the erythrocytes were centrifuged and washed three times with PBS and followed by post-fixation with 1% osmium tetroxide for 30 min at room temperature. After washing with PBS several times, the samples were dehydrated serially with 50, 70, 90, and 100% ethanol. After drying and coating with gold, the images were observed on scanning electron microscope (JEOL, Tokyo, Japan). For confocal microscopy, 200 l of erythrocytes suspension was filled and attached for 1 h to 8-chambered coverslip (NUNC, Roskilde, Denmark), which has been coated with 0.1 mg/ml poly-L-lysine. After washing coverslip three times with Ringer’s solution containing 2% BSA, erythrocytes were stained with Ringer’s solution containing anti-glycosphorin A-FITC for 30 min and washed three times again. Then, erythrocytes were incubated with vehicle (Ringer’s solution) or Pb²⁺ and then observed using confocal microscopy equipped with argon laser (Leica, Wetzlar, Germany). Excitation and emission filters were set at 488 and 550–600 nm, respectively.

Measurement of Intracellular ATP Level

After incubation with lead, erythrocytes were washed and resuspended in Ringer’s solution containing 1mM CaCl₂. The aliquot was mixed vigorously with 10% TCA solution and TAE buffer (100mM Tris-acetate, 2mM EDTA, pH 7.8) and then cooled in ice for 20 min. The sample was centrifuged and the aliquot of resultant supernatant was mixed with cold TAE buffer. Samples were incubated with luciferin/luciferase assay in Luminoskan (Labsystems, Espoo, Finland) for 10 min at room temperature and then observed using confocal microscopy.

Measurement of Intracellular Calcium Level

For detecting intracellular calcium increase, erythrocytes were loaded with 3pM Fluo-4 AM for 1 h at 37°C in the dark. Subsequently, the cells were washed twice and then resuspended in Ringer’s solution to a final concentration of 5 x 10⁷ cells/ml with 1mM of CaCl₂. After erythrocytes were incubated with...
Pb²⁺ for 4 h at 37°C, samples were analyzed on the flow cytometer FACScalibur (Becton Dickinson). Data from 10,000 events were collected and analyzed using CellQuest Pro software.

**Measurement of In Vitro Erythrophagocytosis**

Human leukemia cell lines, THP-1, were purchased from the American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin at 37°C under 5% CO₂ atmosphere. THP-1 cells were prepared as described by Dasgupta et al. (2008). THP-1 cells were cultured (1 × 10⁶ cells per well) in 60 cm² dish overnight with PMA 600 ng/ml to differentiate to macrophage, followed by coincubation for 18 h with vehicle- or Pb²⁺-treated human erythrocytes (2 × 10⁷ cells per well) and then observed under phase contrast microscopy (Olympus IX70, Japan). For coincubation with macrophages, chemical-treated erythrocytes were washed and resuspended with RPMI 1640. For flow cytometric analysis, erythrocytes were loaded with 10 μM of CFDA for 30 min before coincubation. After the coincubation, macrophages were harvested and washed several times to remove unattached erythrocytes. Samples were analyzed on the flow cytometer FACScalibur (Becton Dickinson). Data from 10,000 events were collected and analyzed using CellQuest Pro software.

**In Vivo Experiments**

**Detection of PS exposure following lead administration in rats.** All the protocols used in *in vivo* experiments were approved by the Ethics Committee of Animal Service Centre at Seoul National University. Male Sprague-Dawley rats (Samtako Co., Korea) weighing 200–250 g were used in all experiments. Before the experiments, animals were acclimated for 1 week. Food and water were provided *ad libitum*. At the time of experiment, rats were randomly grouped for control (DW), 10, and 50 mg/kg of Pb²⁺ doses. Four hours after lead was administered per oral, blood was collected from the abdominal aorta using 3.8% trisodium citrate as anticoagulant. An aliquot of blood sample was diluted 200-fold with the following buffer (10 mM HEPES-Na, 136 mM NaCl, 2.7 mM KCl, 2.0 mM MgCl₂, 1.0 mM NaH₂PO₄, 5.0 mM dextrose, 5 mg/ml BSA, 2.5 mM CaCl₂, pH 7.4) and was stained with annexin V-FITC for 15 min in the dark. PS exposure was measured as described above.

**Measurement of hematocrit and hemoglobin.** Four groups of male rats were given 0, 50, 250, and 1000 ppm lead acetate in drinking water for 4 weeks. At the terminal sacrifice, blood was collected from the abdominal aorta using dipotassium EDTA as anticoagulant. Hematocrit and hemoglobin content were determined using the CELL-DYN 3500 (ABBOTT, Abbott Park, IL) within 24 h.

**Hematoxylin and eosin staining and Prussian blue staining of organs.** Liver and spleen were obtained surgically after blood collection, weighed, and fixed with 10% formalin. The tissue specimens were cut into 4-μm thick sections and stained with hematoxylin and eosin (H&E) and ferricyanide. After counterstaining with nuclear fast red solution, the ferricyanide-stained slides were dehydrated and mounted. The slides were observed with bright field microscope (Olympus CX41).

**Statistical Analysis**

The means and SEs of means were calculated for all treatment groups. These data were subjected to one-way ANOVA followed by Duncan’s multiple range test or Student’s *t*-test to determine which means were significantly different from the control. In all cases, a *p* value of < 0.05 was used to determine significant differences.

**RESULTS**

To examine if the exposure to lead can induce PS externalization in erythrocytes, freshly isolated human erythrocytes were incubated with low concentrations of Pb²⁺ (0.1–0.5μM lead acetate) or deionized water for 24 h at 37°C, and PS exposure was measured by flow cytometer. Pb²⁺ treatment increased PS exposure in a concentration- and time-dependent manner, as determined by increased annexin V-FITC binding (Figs. 1A–C). Notably, extremely low concentrations of Pb²⁺ (as low as 0.1μM) could significantly induce PS exposure after a long-term incubation. Along with PS externalization, erythrocytes displayed abnormal echinocytic shapes with protruded surface as observed in confocal microscopy (Fig. 1D). The shape changes in erythrocytes induced by Pb²⁺ were further confirmed in scanning electron microscopy where normal discocytic shapes transformed into echinocytic erythrocytes and further into spherocytes progressively (Fig. 2A).

Shape changes from normal discocytes into spherocytes usually accompany a substantial loss of membrane surface through vesiculation and subsequent MV generation. As shown in Figures 2B and 2C, the exposure to Pb²⁺ resulted in MV generation in a concentration- and time-dependent manner. In addition, generated MV also expressed PS on their outer membrane, which can be recognized and engulfed by macrophages (Willekens et al., 2005).

PS exposure and MV generation can be mediated by the alteration in activities of aminophospholipid translocases (Chung et al., 2007; Lim et al., 2010). We measured the activities of flippase and scramblase, representative enzymes involved in aminophospholipid translocation, after Pb²⁺ treatment. As shown in Figure 3A, flippase which recovers the exposed PS into inner membrane was inhibited by Pb²⁺. However, scramblase which disrupts membrane lipid symmetry was not affected by Pb²⁺, even at high concentrations (Fig. 3B). Flippase activity can be attenuated by the depletion of intracellular ATP (Daleke and Lyles, 2000). Luciferin/luciferase assay demonstrated that the exposure to Pb²⁺ for 24 h depleted intracellular ATP significantly (Fig. 3C), which matched well the inhibition of flippase. Consistently, with the lack of effect on scramblase, the intracellular Ca²⁺, a trigger for scramblase activation, was not affected (Fig. 3D).

Tissue macrophages can recognize and engulf PS-exposed erythrocytes. To investigate if the exposure to Pb²⁺ could induce erythrophagocytosis, Pb²⁺-treated human erythrocytes were coincubated with THP-1 cells that had been differentiated into macrophage with PMA. As shown in Figure 4A, macrophages were attached to Pb²⁺-exposed erythrocytes. Flow cytometry analysis demonstrated more clearly that macrophages engulfed Pb²⁺-exposed erythrocytes (Fig. 4B).

PS-exposed erythrocytes could be easily sequestered from systemic blood flow by spleen and liver. The effects of Pb²⁺ exposure on PS exposure and splenic sequestration of erythrocytes were examined in rats *in vivo*. Prior to *in vivo* experiments, we confirmed that Pb²⁺-induced PS exposure and MV generation were well reproduced in rat erythrocytes (Figs. 5A and B), although rat erythrocytes were less sensitive than human erythrocytes. Significant decrease in intracellular
ATP was also observed (Fig. 5C) suggesting that Pb\(^{2+}\)-induced PS exposure through ATP depletion in rat erythrocytes as in human. Consistently, with these results, the oral administration of 10 or 50 mg/kg of Pb\(^{2+}\) to rats induced PS exposure \textit{ex vivo} indeed (Fig. 5D). To examine the long-term effects of Pb\(^{2+}\) exposure on erythrocyte clearance, drinking water containing 0, 50, 250, and 1000 ppm lead acetate was supplied to rats subchronically for 4 weeks. Hematocrit and hemoglobin level, indicators of erythrocyte count, were significantly decreased by the exposure to Pb\(^{2+}\) (Figs. 6A and B). The relative organ weights of both liver and spleen increased significantly (Fig. 6C). Most strikingly, Pb\(^{2+}\) exposure from the doses as low as 250 ppm induced a clear sign of iron accumulation in the spleen (Fig. 6D), indicating that Pb\(^{2+}\) accelerated erythrocyte clearance through erythrophagocytosis indeed.

**DISCUSSION**

In the present study, we demonstrated that Pb\(^{2+}\) could increase splenic sequestration of erythrocytes through PS exposure and erythrophagocytosis. Pb\(^{2+}\)-induced PS exposure was mediated through the inhibition of flippase by the depletion of intracellular ATP. Abnormal shape and MV generation, other triggers for the erythrophagocytosis by macrophage, were also observed in the Pb\(^{2+}\)-treated erythrocytes. We could also show that human macrophages could successfully recognize and phagocytosis lead-exposed erythrocytes. These observations were confirmed in rat \textit{in vivo} through \textit{ex vivo} PS exposure and iron accumulation in the spleen, suggesting that Pb\(^{2+}\)-induced PS exposure in erythrocytes can provoke erythrocyte clearance and ultimately anemia.

Anemia is one of the most well-known toxic health effects associated with lead exposure. Various mechanisms have been suggested for Pb\(^{2+}\)-associated anemia including interference with iron transport, shortening of erythrocyte life span and inhibition of the globulin synthesis, and ribosomal RNA degradation (Ichiba \textit{et al.}, 1992; Kim \textit{et al.}, 1995), but the impairment of heme metabolism has been regarded as a major contributing factor. Lead inhibits several enzymes participating in the heme synthesis such as ALAD and heme chelatase. Especially, ALAD is very sensitive to lead, of which IC\(_{50}\) was estimated to be around 1.9 \textmu M (Davis and Avram, 1980). Because of this sensitivity, the inhibition of ALAD and subsequent impairment of heme production is suggested to play a major role in lead-associated anemia. However, direct evidence supporting the relationship between ALAD inhibition and anemia was not provided, casting a doubt on the role of the inhibition of ALAD or heme synthesis in lead-associated anemia. Signifying this doubt, although the inhibition of ALAD is observed at very low level of BLL ranging 2.7–2.9 \textmu g/dl (Murata \textit{et al.}, 2003), anemia was reported at much lower levels.
higher concentrations (> 62 µg/dl). In addition, people with ALAD2 polymorphism, which has a higher affinity to lead than those with ALAD1 polymorphism, are known to exhibit lower occurrence of anemia (Sakai et al., 2000; Su¨zen et al., 2003). In these reports, ALAD is suggested to work as an endogenous chelating agent for lead, detoxifying and inactivating blood lead from manifesting toxicity. Similar to ALAD, low doses of Pb²⁺ (0.1–0.5 μM) can induce PS exposure, shape changes, and erythropagocytosis after an extended time of exposure in vitro. This concentration is in a proximate range to the BLL defining lead poisoning (> 10 μg/dl, 0.5 μM). Most importantly, subchronic exposure to Pb²⁺ through drinking water could increase PS exposure and erythrocyte clearance in vivo (Fig. 6). These results suggest that increased clearance of erythrocytes mediated through PS exposure by lead exposure can contribute to anemia, indeed.

PS exposure on erythrocytes is a strong phagocytic signal of apoptosis to tissue macrophages. PS is recognized by various PS receptor and glycoprotein-like lactadherin, TIM-1 and TIM-4 (T-cell immunoglobulin- and mucinomain-containing molecule). Tissue macrophages recognize and uptake this complex (Fens et al., 2008; Kobayashi et al., 2007). Role of PS exposure and shape changes of erythrocytes in anemia was well established in disease states such as sickle cell anemia (de Jong and Kuypers, 2006; Kuypers et al., 1998; Lang et al., 2007). Recently, we demonstrated that a drug can also induce anemia through PS exposure and subsequent erythropagocytosis (Noh et al., 2010). However, there is no report concerning environmental toxicant-induced erythropagocytosis to our best knowledge. Our study demonstrating that lead-induced PS exposure can contribute to anemia further substantiates the important role of PS exposure and subsequently increased erythrocyte clearance in the chemical-induced anemia.

Depletion of ATP is shown to underlie Pb²⁺-induced PS exposure. Pb²⁺ inhibits enzymes associated with glycolysis, which provides erythrocytes with ATP (Baranowska-Bosiacka and Hlynczak, 2003). Glyceraldehyde 3-phosphate dehydrogenase (Grabowska and Gumin’ska, 1996) and pyruvate kinase (Paglia et al., 1975) are the representative enzymes that are inhibited by Pb²⁺. In addition, decreased heme synthesis through ALAD can also affect energy...
metabolism, which might impair ATP synthesis. Nakao and his colleagues discovered decreased ATP content led morphological transformation of erythrocytes from discocytes to echinocytes (Nakao et al., 1960). ATP sustains non-equilibrium dynamic fluctuations in the erythrocyte membrane, which is critical in the maintenance of normal biconcave shapes (Park et al., 2010). Therefore, we believe that the depletion of ATP may explain lead-induced PS exposure on erythrocytes.

Although Pb2+ induced significant level of PS exposure in human erythrocytes in vitro at the low concentrations from 0.1 to 0.5 μM (2–10 μg/dl), erythropagocytosis and anemia were manifested at relatively high Pb2+ exposure level (250–1000 ppm drinking water). With the subchronic exposure to drinking water containing 250–1000 ppm Pb2+, BLL could be increased up to 9.1 ± 1.7 to 22.4 ± 2.3 μg/dl (Lee et al., 2006). The discrepancy of “potency” between in vitro human erythrocytes and in vivo rat study could be explained by the species difference. Rat erythrocytes were less sensitive to lead-induced PS exposure than human erythrocytes in vitro. At 2 μg/dl Pb2+, a significant level of PS exposure could be detected in human erythrocytes (Fig. 1B) but not in rat erythrocytes (Fig. 5A). Only from 10 μg/dl lead, rat erythrocytes showed a significant level of PS exposure. The level of difference in potency is estimated to be more than fivefold. Considering this species difference, it is understandable that BLL of 9–22 μg/dl (with 250–1000 ppm lead-contaminated drinking water) should be required to induce lead-induced PS exposure in erythrocytes and erythropagocytosis in rats in vivo. In the same context, however, it is plausible that human may be more sensitive to lead-induced erythropagocytosis through PS exposure although further study is required to prove it.

In conclusion, we demonstrated that low dose lead can induce PS exposure and MV generation through ATP depletion. Most importantly, we found that subchronic exposure to lead increased erythrocyte clearance indeed. We believe that our study will provide a new insight into the role of PS exposure and erythropagocytosis in lead-induced anemia.

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REFERENCES


FIG. 6. In vivo hematocrit and hemoglobin change and relative organ weight change and increase of erythropagocytosis by Pb2+ treatment in SD rats. After drinking water administration of saline, 50, 250, and 1000 ppm of Pb2+ for 4 weeks, the amount of (A) hematocrit and (B) hemoglobin was measured. (C) Liver and spleen were dissected and weighed, followed by fixed for Prussian blue staining. (D) Representative microscopic photograph of spleen from control, 50, 250, and 1000 ppm of Pb2+-treated group were shown. The original magnifications were 400-fold. Values are the mean ± SEM of four to eight independent experiments. *Significantly differences from the control group (p < 0.05).


